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Procedure for quantitative determination of viral particles having a cholesterol-containing envelope

The invention relates to a procedure for quantitative determination of viral particles having a cholesterol-containing envelope, using the fluorescent dye filipin.

Background

Virus quantification

Quantifying total viral particles is a relatively involved procedure compared to determining the number of infectious viral particles. Detection of retroviral particles in cell culture supernatants is used, on the one hand, to exclude the possibility of contamination with retroviruses and, on the other hand, to check the quantity and quality of virus production by packaging cells. Contamination with retroviruses is usually detected by means of the activity of viral reverse transcriptase in the supernatant [85, 106] (a literature list, including bibliographic details, will be found at the end of the description). There are various methods available for quantifying the production of total viral particles . Besides the afore-mentioned activity of reverse transcriptase, determination of viral "strong-stop" cDNA, electron microscope investigations and immunohistochemical staining of proteins on the virus surface are used for quantifying total particles [7, 84, 85, 103].

Those methods can be categorised, according to their principle, into two types. On the one hand, they consist of indirect quantification methods wherein the number of viral particles is inferred from viral enzyme activities or from the nucleic acid content. On the other hand, there are direct methods, serving to make the viral particles visible. The first category includes detection of viral RNA, of viral "strong-stop" cDNA and of the activity of viral reverse transcriptase [84, 85, 143, 144]. Electron microscope investigations and immunohistochemical staining of viral envelope proteins on the surface of the virus are included in the second category of methods used [103, 7]. The advantages of the indirect methods lie in the fact that they are relatively simple to perform and a large number of samples can be quantified rapidly. The disadvantage of that kind of particle number determination lies in the fact that it provides only an approximate idea of the number of

viruses released and, as has been shown in the context of investigations relating to the invention, factors specific to the cell type are in some cases required to calculate the total particle numbers. It is, for example, usually assumed that 1 % of infectious retroviruses contains "strong-stop" cDNA, irrespective of the cell line and production conditions. However, the data presented together with the invention show that the number of infectious viruses as a proportion of the cDNA-containing particles varies in dependence upon the type of production cell line. For the murine fibroblast cell line NIH3T3, it has been possible to demonstrate, in a number of experiments, that approximately half the infectious particles contain "strong-stop" cDNA. That contradicts the data of other study groups, which have shown that about 1 % of total particles are infectious [84, 149]. In order to quantify total viral particles exactly using this method, therefore, it is necessary to take into account a factor specific to the cell type. That factor can, however, be determined only using methods that, like the direct methods, are capable of detecting all viral particles. However, electron microscope investigations, for example, require a not inconsiderable outlay on apparatus and materials. The immunohistochemical staining procedures developed by Pizzato *et al.* [7] can, in contrast, be performed relatively rapidly and simply, but they do, in some cases, require a considerable outlay in optimising the staining procedures and they are dependent upon the existence of a corresponding antibody to viral surface proteins.

Principle of filipin staining

When retroviruses are formed and released, the viral membrane is formed from the plasma membrane of the production cell. In terms of its composition, that viral envelope is similar to the membrane regions in which budding of the virus occurred [46], with one of the constituents, besides proteins, sphingolipids and glycolipids, being cholesterol. The latter lipid, to which filipin binds specifically, is an essential constituent of eukaryotic cells. Filipin has been isolated from the culture supernatant of *Streptomyces filipinensis*; it consists of a 35-membered lactone ring and accordingly belongs to the group of the polyene macrolide antibiotics [145]. Earlier studies have shown that filipin forms a complex with the cholesterol of the external envelope of retroviruses and other enveloped viruses [146, 147], the viral membranes being used in those studies for investigation of protein-lipid interactions with the aid of electron micrographs.

The invention aims to provide a procedure or method for quantitatively determining viral and bacterial particles that can be carried out rapidly and simply but is nevertheless reliable.

That aim is achieved in accordance with the invention by a method of quantifying viral or bacterial particles having a cholesterol-containing envelope, wherein the particles are stained with a fluorogenic polyene macrolide and the fluorescence signals of the individual particles are then quantitatively determined.

In accordance with the invention, the method can be applied to retroviruses, orthomyxoviruses, paramyxoviruses, arteriviruses, togaviruses, bunyaviruses, rhabdoviruses, filoviruses, arenoviruses, coronaviruses, herpesviruses, flaviviruses, hepadnaviruses, poxviruses or iridoviruses.

In accordance with the invention, the method can be applied to, especially, HIV, measles virus, influenza virus, murine leukaemia virus or mycoplasmas.

In the method according to the invention, the number of particles can be determined by counting fluorescent particles under a fluorescence microscope.

Furthermore, in the method according to the invention, filipin can be used as the polyene macrolide.

Furthermore, in the method according to the invention, the filipin fluorescence can be excited at a wavelength of 387 $\pm$ 14 nm and the counting can be carried out at the emission wavelength of 450 $\pm$ 29 nm.

Furthermore, in the method according to the invention, for quantitative determination, the number and/or concentration of fluorescent particles can be compared to the known number and/or concentration of specified fluorescent particles.

Furthermore, in the method according to the invention, for comparison, fluorescent particles can be specified that are from 0.5 times to twice as large as, especially about the same size as, the particles being quantified.

Finally, in the method according to the invention, for comparison, inert fluorescent particles can be specified.

The method of quantifying viruses developed in the context of the present invention combines the advantages of antibody-independent staining of the viral or bacterial membrane and quantification with the aid of a fluorescence microscope.

The interactions between cholesterol and filipin have not hitherto been used for quantifying viral particles by means of fluorescence using a fluorescence microscope, one of the reasons probably being that the possibility of using high-quality lenses of about 1000x magnification for making viruses or bacteria visible has been underestimated.

The invention relates further to a kit of parts for quantifying viral or bacterial particles having a cholesterol-containing envelope, which comprises
- a fluorogenic polyene macrolide and
- (as optional constituent) fluorescent particles as reference standard.

In the kit of parts according to the invention, the reference standard can be present in an aqueous medium.

Furthermore, in the kit of parts according to the invention, the fluorescent particles of the reference standard can be inert particles.

Furthermore, in the kit of parts according to the invention, the fluorescent particles of the reference standard can be from 0.5 times to twice as large as, and especially about the same size as, the particles being quantified.

Finally, the kit of parts according to the invention can be characterised by filipin as the polyene macrolide.

The invention finally relates also to the use of a fluorogenic polyene macrolide, especially filipin, for quantifying viral or bacterial particles having a cholesterol-containing envelope.

The invention is illustrated, without limitation, in greater detail hereinbelow, with reference to Examples and Figures.

Figure 1 shows: labelling of the viral membrane of 4070A MLV's with filipin. a) 4070A MLV produced by NIH3T3 after transfection with the 4070A MLV provirus and the retroviral vector pLEIN. After fixing on glass slides by means of Polybrene, the viruses were labelled with filipin; b) negative control; supernatant of mock-transfected NIH3T3; c) Texas Red-labelled beads 100 nm in size. The beads were likewise fixed on glass slides using Polybrene and serve as a reference for the size of the viral particles, which are about 100 nm in size, and as a standard for quantification; fluorescence micrographs, original magnification 1000x (oil immersion lens).

Staining of viral particles, using the example of 4070A MLV

The fluorogenic properties of filipin are utilised for quantifying viral particles. Viruses labelled with filipin can be made visible and quantified under a fluorescence microscope (excitation 387 nm; emission 450 nm) (see Figure 1). Quantification of the physical viral particles is carried out by direct comparison with Texas Red-labelled beads added in a known concentration to the virus-containing supernatant before fixing (see Methodology chapter, Quantification section).

Advantages of filipin staining over other methods of quantification

Filipin staining has a number of advantages: it can be performed simply and rapidly (taking less than 2 hours). Comparison with the quantification of "strong-stop" cDNA shows that it is up to 40 times more sensitive (compare Table 1). Because of its specificity for cholesterol, the method does not require any lengthy optimisation as is frequently necessary in the case of immunohistochemical staining procedures, and it is not dependent on virus-specific antibodies, which is advantageous, in particular, in the case of those viruses for which antibodies are not available. The viral particles can be made visible using a conventional fluorescence microscope of a kind that can be found in any biological establishment. The sole condition is that 1000x magnification is possible and an appropriate filter set is available, both being possible, however, with a simple microscope outfit. A further advantage of this particle staining procedure is that it can be used generally

for any type of virus that has a cholesterol-containing envelope (e.g. HIV, measles virus, influenza virus).

Table 1: Comparison of particle number determination using cDNA measurement and filipin labelling of 4070A MLV.

Cell line	cDNA particles [particles/10^6 cells/24 h]	filipin-labelled particles [particles/10^6 cells/24 h]
NIH3T3	3.1×10^7	1.5×10^8
BHK-A	3.7×10^6	5.8×10^7
BHK 21B	6.0×10^6	2.6×10^7
TE FlyA7	8.2×10^5	3.2×10^7

Determination of the variance of quantification of filipin-labelled viral particles

In order to use filipin labelling for quantifying physical viral particles, it must be ensured that the analyses are reproducible. For that purpose, nine viral 4070A MLV samples were stained, with duplicate determinations being carried out, and each individual staining was quantified. The average deviation from the mean was calculated from the data. The average deviation was 12.5 %.

Methodology

Staining of viral membranes

For systematically quantifying viruses enveloped by the plasma membrane of the host cell, there has been developed a staining method that is not dependent on antibodies because the cholesterol of the viral membrane is fluorescence-labelled in targeted manner. Filipin is an antibiotic which acts on the membrane sterols of eukaryotic cells, leading to changes in membrane permeability. By virtue of its fluorescence properties and specific binding to cholesterol, it can be used for staining cholesterol-containing membranes.

For staining, 195 µl of virus-containing cell supernatant (filtered, 0.45 µm) were mixed with 4 µl of Polybrene (0.4 mg/ml) and 1 µl of Texas Red-labelled fluorescence particles (Molecular Probes, diameter 100 nm, 3.6 x 10^7/µl) and incubated for 1 hour at room temperature in chamber slides provided with 8 recesses. The samples were then washed three times with 1XPBS and were incubated with glycine (1.5 mg/ml) for 10 min and then with filipin (0.05 mg/ml) for 30 min. The specimens were washed three times with 1XPBS; fluorescence enclosure medium (Dako) was poured over the specimens and they were sealed with a glass coverslip.

1XPBS: 140 mM NaCl; 27 mM KCl; 7.2 mM $NaHPO_4$; 14.7 mM KH_2PO_4; pH 6.8 – 7.0 as 10X stock solution from GibcoBRL; solution for use diluted 1:10 with distilled water

Detection of stained viruses

The number of viral particles in a virus-containing cell supernatant is determined with the aid of fluorescence micrographs (1000x magnification, 100x oil immersion lens). Visualisation of the fluorescent particles was carried out using filters of the wavelengths 595 / 615. The filipin signals can be detected with the aid of a fluorescence microscope (Filter Set XF113 from Omegafilters, excitation 387 nm (28), emission 450 nm (58)).

Quantification of filipin-labelled viral particles

For quantification, micrographs are taken, from the same specimen recess, of the filipin-labelled viruses and the fluorescence-labelled beads. For a statistically significant evaluation of quantification, at least seven micrographs should be taken (both for viruses and for beads). The viruses and beads visible on the images are counted. Determination of the concentration of viruses is carried out using formula 1. Calculation is made possible by using beads in a known concentration.

$$\frac{C_{beads} \times A_{virus}}{A_{beads}} = C_{virus} \qquad (1)$$

C_{beads} – concentration of beads (number/ml)

C_{virus} – concentration of viruses
A_{beads} – number of beads per image
A_{virus} – number of viruses per image

In summary, in accordance with the invention, a new method has been developed for quantifying viruses that have a cholesterol-containing membrane. The method consists of an antibody-independent staining procedure wherein the cholesterol molecules of the viral membrane are specifically labelled by the antibiotic filipin. Filipin has fluorogenic properties and can be visualised using a filter set for bfp (blue fluorescence protein; excitation at 387 nm, emission at 450 nm). As a result of that fluorescence, the viruses can be made visible using a fluorescence microscope at 1000x magnification. Quantification is carried out by direct comparison with fluorescence-labelled (e.g. Texas Red-labelled) particles 100 nm in size, the concentration of which is known.

Definitions, abbreviations

4070A MLV: murine leukaemia virus which is capable of infecting the cells of a large number of organisms

reverse transcriptase: enzyme for the transcription of RNA into DNA

"strong-stop" cDNA: formed by reverse transcriptase in viral particles of 4070A MLV and used for quantifying viral particles

immunohistochemical staining procedures: fluorescence-labelled antibodies bind to specific surface structures and can be made visible under a fluorescence microscope

beads: term for spherical particles

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